Universidade de Aveiro Departamento de Biologia
2009

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Cultura de Tecido Ovárico Humano Congelado: efeito da Activina A e GDF9

Culture of Human Cryopreserved OvarianTissue: effect of Activin A and GDF9

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica du Docteur Catherine Poirot, Professeur de la Faculté de Médecine, Paris VI, Pierre et Marie Curie e da Doutora Maria de Lourdes Gomes Pereira, Professora Associada com agregação do Departamento de Biologia da Universidade de Aveiro

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agradecimentos

Au Professeur Catherine Poirot, pour m'avoir fais confiance encore une fois et de continuer à m'orienter dans mon travail, à sa disponibilité et son enthousiasme pour mon travail. Merci encore de me permettre de travailler avec vous et votre équipe.

Aux Docteurs Marie Prades et Benoit Schubert, pour leur amitié et leur soutien.

A Aurélie, Leslie, Monia et Nicolas pour tous ces moments de détente partagés.

A toute l'équipe de l'UF de Biologie de la Reproduction de la Pitié-Salpêtrière pour m'avoir aussi bien accueille au sein de leur petite «famille».

A la Fondation Martine Midy qui a permis la réalisation de ce projet en me finançant.

A ma sœur de coeur et d'esprit, Sandrine, pour me tolérer encore chez elle malgré ma mauvaise humeur, de comprendre même mes silences, pour toutes les heures passées à m´écouter et à me conseiller.

Aos meus pais, por tudo o que fizeram para que eu pudesse seguir os meus sonhos e por me terem encorajado mesmo estando tão longe.

Aos meus irmãos, obrigada por estarem sempre presentes e pelos abraços que me dão força para voltar.

Ao Renato por nunca se negar a dar resposta às minhas perguntas, mesmo as menos pertinentes.

Ao Jacinto um obrigada muito especial pela Amizade, tolerância e força que me tem dado para a concretização deste projecto.

palavras-chave **Crioconservação/ Cultura tecido Ovárico/ Activina A/GDF9**

resumo

A crioconservação de tecido ovárico é um dos métodos utilizados para preservar a fertilidade de mulheres em risco de menopausa precoce, devido a tratamentos gonadotóxicos.

O tecido ovárico crioconservado contém essencialmente oócitos imaturos, não fecundáveis, sendo por isso necessário induzir a sua maturação. A maturação dos oócitos contidos no tecido ovárico crioconservado pode ser realizada transplantando o tecido (*in vivo*) ou procedendo à sua cultura (*in vitro*), tema deste trabalho.

Este trabalho foi desenvolvido para avaliar a morfologia e a evolução dos folículos ováricos contidos no tecido, recorrendo a três marcadores de qualidade celular [dois marcadores da apoptose (p53 e Bcl2) e um marcador de proliferação celular (Ki67)], sem matriz de suporte, em três meios de cultura distintos durante 14 dias.

Este estudo comparativo foi realizado utilizando um meio de cultura base, também usado como grupo de controlo, ao qual se adicionaram, individualmente, duas proteínas pertencentes à Tranforming Growth Factors superfamily, GDF-9 e Activina A.

Neste trabalho, verificou-se uma diminuição da população folicular ao longo da cultura para os três meios utilizados.

No entanto, a maior proporção de folículos degenerados foram observados no grupo em cultura com Activina A contrapondo com o grupo em cultura com GDF-9 onde se observou a menor percentagem de folículos degenerados.

Nos três meios de cultura utilizados apenas se observaram três tipos de folículos (primordial, early primário e primário).

Verificou-se uma marcação positiva para os marcadores p53 e Ki67 em todos os meios e ao longo da cultura. Para o marcador Bcl2 não foi observada nenhuma célula da granulosa marcada positivamente.

Com este trabalho verificamos uma melhor taxa de sobrevivência no grupo GDF-9, assim como um maior número de folículos e células da granulosa nos primeiros 10 dias de cultura.

keywords **Cryopreservation/Ovarian tissue culture/Activin A/GDF9**

abstract

Cryopreservation of ovarian tissue is one approach to preserve fertility of young women who are at risk of premature ovarian failure, due principally to a gonadotoxic treatment.
The cryopreserved ovarian tissue has essentially immature oocytes, which need to be maturating for ulterior fertilization.
Oocyte maturation can be achieved by transplantation or by maturation in vitro, which was the subject of this work.
The present work was developed to evaluate the morphology and evolution of follicles using three markers of cellular quality [two apoptotic markers (p53 and Bcl2) and one proliferating marker (Ki67)] during 14 days of culture for three different culture media, without matrix support.
For this comparative study we used a serum free medium (control group) to which we added two proteins from the Transforming Growth Factors superfamily Activin A and GDF-9.
A diminution of the follicular population was observed throughout the culture and for all the culture media. However, the higher proportion of degenerating follicles (increasing with the time of culture) were observed at the Activin A group (A) and GDF-9 group (G) representing the lower proportion.
For the three culture media only three type of follicles were seen (primordial, early primary and primary).
All GCs in the three culture media were negative for Bcl2 immunostaining. .
Positive marking were observed for the apoptotic marker (p53) and for the proliferating marker (Ki67) in all culture.
This study indicate a better survival in the group cultured with GDF-9, a higher number of follicles and cells of the granulosa in the first 10 days of culture

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ABREVIATIONS

AMH	Anti-Mulerian Hormone
ART	Assisted Reproductive Technology
BFGF	Beta Fibroblast Growth Factor
BMP	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
DBA	Diaminobenzidine Tetrahydrochloride
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
EG	Ethylene Glycol
FCS	Fetal Calf Serum
FD	Follicular Development
FSH	Follicular Stimulating Hormone
GC	Granulosa Cell
GDF-9	Growth Differentiation Factor 9
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
HES	Haematoxylin, Eosine and Safranin
HRP	Horsedarish Peroxidase
ICSI	Intra Cytoplamisc Sperm Injection
IGF I	Insulin like Growth Factor I
IGF II	Insulin like Growth Factor II
IVF	In Vitro Fertilization
KL	Kit-ligand
LH	Luteinizing hormone
MI	Methaphase I
MII	Methaphase II
mRNA	Messenger Ribonucleic Acid
PGC	Primordial germ cells
POF	Premature ovarian failure
PrOH	PropanediolL
RNA	Ribonucleic Acid
TGF – ß	Transforming Growth Factor ßeta

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INTRODUCTION

Women have a finite number of follicles that sustain ovarian function until the menopause. Any situation affecting the follicles pool can induce a premature ovarian failure. This failure can be caused by genetics disorders and more commonly as a consequence of anticancer treatments exposure, being extremely important to propose methods to preserve their fertility.

One approach to preserving the potential fertility of young women who risk losing their follicles reserve is the cryopreservation of ovarian tissue containing immature oocytes.

The cryopreserved tissue can be transplanted into the pelvic cavity (orthotopic) or in a heterotopic site such the abdominal wall or the forearm. However in some types of cancer the transplantation has a high risk of reintroduce malignant cells, subsequently is very important to study and develop alternative methods such as the maturation *in vitro* of the follicles and their oocyte.

Over the last two decades, culture systems have been developed with the aim of growing follicles from the earliest stage into maturation of the oocyte and fertilization.

In mice offspring it has already been obtained with maturation of primordial follicles and their fertilization *in vitro* but in Human this process reveals being more complex due to the long time needed for the folliculogenesis, the little knowledge of the factors involved in the initiation of the growth of small follicles and the dense structure of the ovarian tissue.

Nevertheless a number of factors produced in the ovary itself, as the Transforming Growth Factor beta family, are known to control the initiation of the growth of small follicles.

At present time, it is known that the GDF-9 plays an important role in the initiation of the growth of small follicles as well as the survival of these throughout the culture while activin A seams to promote the growth and development of secondary follicles to antral follicles.

The aim of our study was to analyze the effects of growth differentiation factor 9 and activin A during the culture of human cryopreserved ovarian tissue, since to date, the published studies show results obtained essentially with culture of fresh ovarian tissue, using morphological characteristics, apoptotic markers (p53, Bcl2) and proliferating marker (Ki 67).

REVIEW OF THE LITERATURE

THE OVARY

The ovary is the reproductive organ in female mammals, it measures about 4 cm long, 2 cm wide, 1 cm of thick and has a dual role of gonad and endocrine gland. Usually, each woman has two ovaries located in the pelvic zone, one in the right side and the other in the left side of the uterus to which they are attached by a fibrous cord called the ovarian ligament.

The outermost layer of the ovaries is the germinal epithelium constituted by simple cuboidal cells. Underneath this, a layer of dense connective tissue constitutes the tunica albuginea and covers the ovarian cortex.

In the cortex, we found the ovarian follicles and between them the stroma, a peculiar soft tissue consisting mainly of connective tissue enriched with blood vessels. The innermost layer of the ovary, called medulla, is essentially loose connective tissue with abundant blood vessels, lymphatic vessels and nerve fibers (Fig.1).

The ovary performs several tasks that include the production, storage and nurturing of the oocytes and segregation of hormones that promote follicle/oocyte maturation and development of secondary sex characteristics.

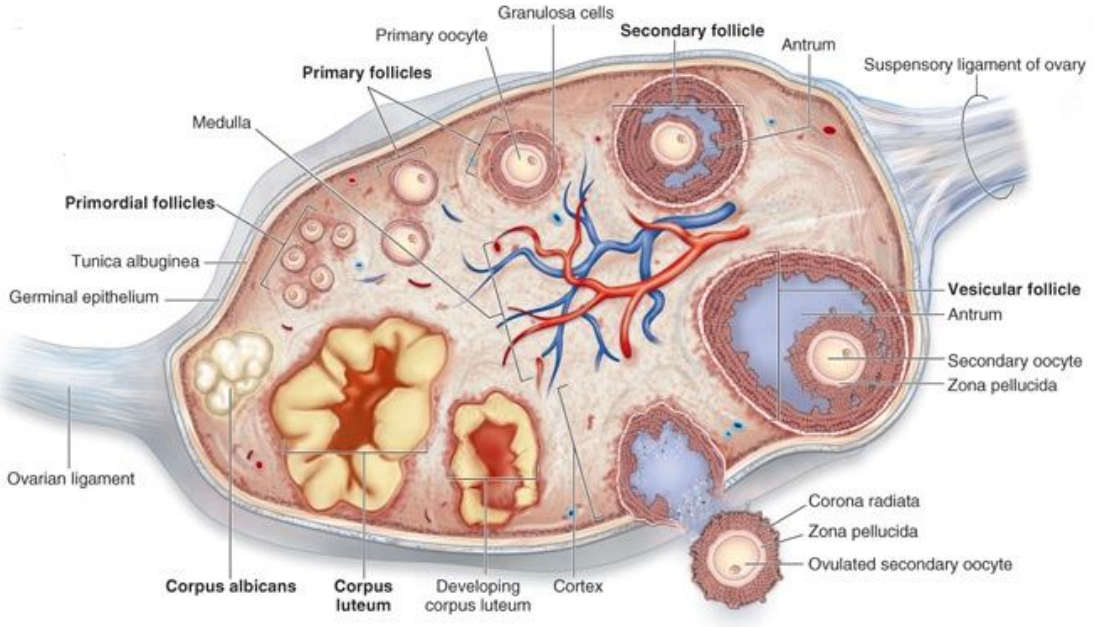

Figure 1: Schematic representation of a ovary showing its internal configuration, and the different types of follicles (Adapted from http://academic.kellogg.cc.mi.us)

OVARIAN RESERVE

In Humans, the follicular reserve is mainly comprised of primordial follicles, containing an oocyte I, and begins to form at the fourth month of fetal life (Baker, 1963), around the 20 weeks of gestation, about seven millions of oocytes are formed in each ovary, and it is believed those are all the oocytes that woman will ever have. At birth the reserve drops to one to two millions and at the onset of puberty only 300 000 to 400 000 remain (Baker, 1963; Faddy et al., 1992; Faddy, 2000). From there, the number of follicles gradually decreases until the age of 37-38 years old, when rapidly drops and ends in menopause several years later with only 1000 follicles in each ovary (Faddy et al., 1992). However, during reproductive life of a woman, only 400 follicles mature and ovulate (Gougeon, 1996), whereas the remainders go through atresia and degenerates.

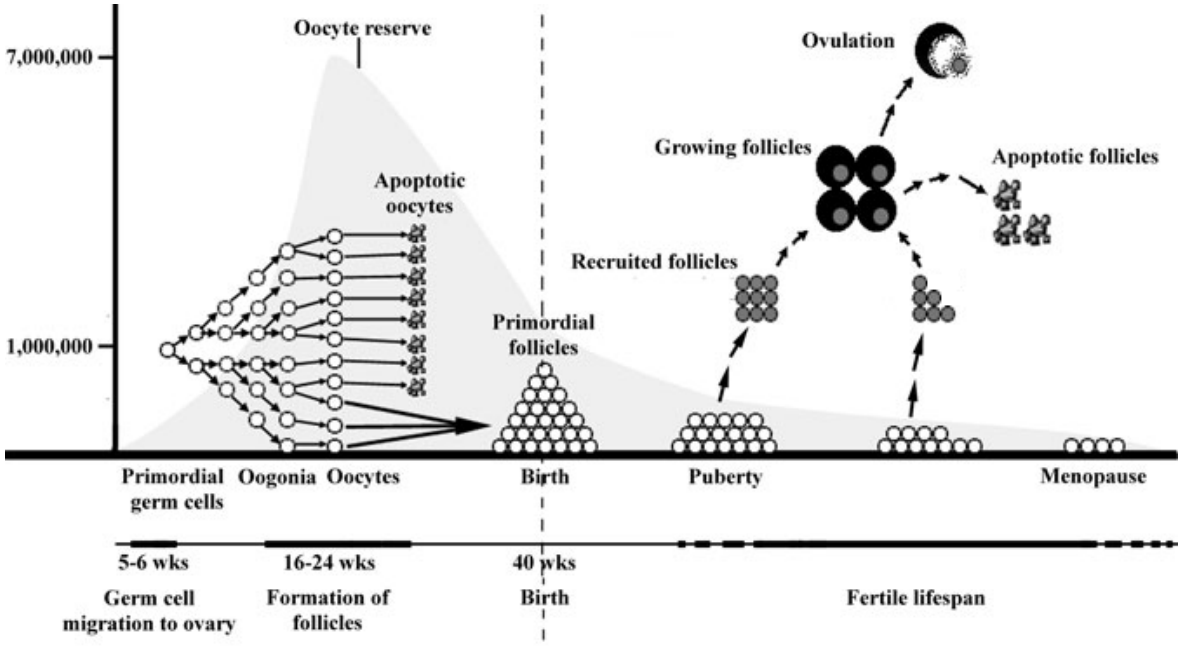

Figure 2 : Diagram of ovarian reserve and outcomes of the formed follicles throughout women life (adapted from Kaipa & Hsueh, *Annu Rev Physiol* 1997, 59:349-63)

The depletion of the ovarian stock occurs as a result of two major processes: atresia, by apoptosis, and entry in growth phase. In the Human the depletion before puberty and after 30 years of age is mainly due to atresia whereas between those ages this follicles loss is due to entrance of resting follicles in growth phase (Gougeon, 1996).

OOGENESIS AND OOCYTE MATURATION

The oogenesis starts with the formation on the oogonia and involves several processes that lead to their development and maturation.

The oogonia are originated from primordial germ cells (PGC) in a common path of gametogenesis in females and males.

At the end of the third week of development PGC's migrate from the primary ectoderm into the yolk sac (Baker et al., 1963). Between the fifth and the sixth weeks, under the influence of cytokines such as Kit-ligand (KL) and transforming growth factor ß (TGFß), these cells continue to migrate until they reach the genital ridge, where they stay and colonize the developing gonads, by mitosis (Motta and Makabe, 1986).

After colonization, around the seventh week of gestation, the sex specific differences appear and the germ cells differentiate into oogonia or spermatogonia according to chromosomal constitution and the environment in which they develop. Henceforth oogonia continue to undergo mitosis and stimulate cells of the adjacent epithelium to form follicular cells (Faddy et al., 1992), but around the eleventh week of development they enter in meiosis and stop in the prophase of the first meiotic division, becoming oocytes I. At the same time, they became enclosed in one layer of pregranulosa cells surrounded by a basal membrane forming a complex called primordial follicle (Gosden and Bownes, 1995). The nucleus of these dormant oocytes I becomes very large and it is called a germinal vesicle (GV).

When the follicle is recruited for the growing pool the oocyte undergoes an important increase of volume from 35µm in primordial follicle to 120µm in a fully developed follicle (Gougeon, 1996; Picton et al., 1998) associated with the follicular growth. However the oocyte I persists in prophase of the first meiotic division until the time of ovulation, when meiosis is resumed and the first polar body is formed and extruded, becoming a fertilizable oocyte. This oocyte is arrested in the metaphase of meiosis II and only completes his second meiotic division after fertilization (Fig 3).

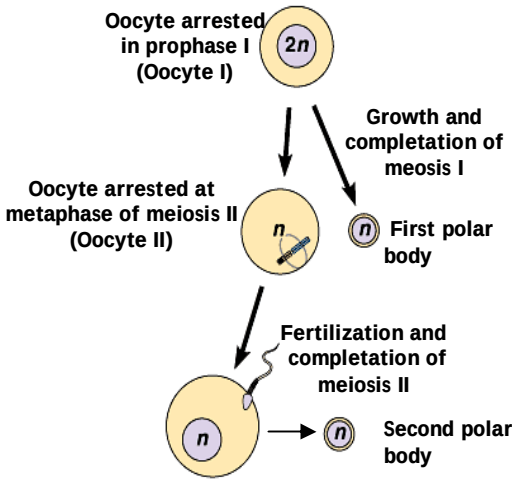

Figure 3 : Meiosis of oogonia (adapted from Pearson education Inc., publishing as Benjamin Cummings)

The fully and competent maturation of the oocyte depends on two crucial events, the long and imperceptible cytoplasmic maturation and the short and clearly observable nuclear maturation.

Cytoplasmic maturation

Cytoplasmic maturation includes accumulation of mRNAs and proteins, cytoplasmic reorganization and epigenetic modifications, necessary to prepare the oocyte for fertilization and normal embryo development, and it is divided in two major events, the acquisition of competence to development (before resumption of meiosis) and the oocyte activation prior to ovulation.

During the oocyte growth, an extensive production and reorganization of the cytoplasm takes place. At this period, the oocyte stores glycogen granules, lipid droplets and proteins likely to provide energy and substrate to the formation of new membranes after fertilization (Picton et al., 1998), the number of mitochondria increases drastically and become more vacuolated, sign of loss activity (Wassarman and Josefewicz, 1978) as well as the number of ribosome, important to the accumulation of mRNA and proteins.

As the oocytes get larger organelles migrate to the periphery of the oocyte, the Golgi apparatus enlarges and becomes active in secreting proteins to the production of the zona pellucida (Mehlmann et al., 1995) and the endoplasmic reticulum forms more cortical distribution where it facilitates the exocytosis of cortical granules by releasing calcium.

During oocyte maturation a large quantity of RNA is stored in the cytoplasm. In a human immature oocyte there is 2 ng of RNA (Neilson et al., 2000) of which 8% is mRNA, a bigger quantity than in a somatic cell (Gosden, 2002). Throughout maturation mRNA is degraded and in a mature human oocyte there is 40% less mRNA than in an immature oocyte (Dobson et al., 2004). The synthesis and expression of the mRNA are controlled by a complex system and depends on when during the maturation process they are needed (Bachvarova, 2002).

The oocyte activation designs the ultrastructural modifications occurred in the oocyte just before the ovulation, which are essential for his capacity to be fertilized and to develop embryo (Hyttel et al., 1997). Thus, in this oocyte, a diminution of the size of the Golgi apparatus, an increase of the number of lipid vesicles and a regrouping of cortical granules is observed (Cran, 1985).

Nuclear maturation

During meiotic division, the genetic material is halved completing two nuclear divisions, but only a single replication of the nuclear DNA.

The immature oocyte is blocked at the diplotene stage of the first meiotic division, and his nucleus is called GV.

The nuclear maturation is characterized by the germinal vesicle break down (GVBD) and the cells enters metaphase I (MI) and progresses with meiosis until it is arrested once again, in the metaphase II (MII) and the first polar body is extruded. At this stage, the oocyte can be fertilized. When the oocyte is fertilized it restarts meiotic division and completes its maturation with the expulsion of a second polar body.

FOLLICULOGENESIS AND STAGES OF FOLLICULAR DEVELOPMENT

Follicles, the basic functional units of the ovary, are comprised in two functional pools, resting and growing follicles. It's from the resting pool that follicles will be recruited for maturation throughout life.

The recruitment is regulated by paracrine and autocrine signals produced in the ovary itself, while proliferation and differentiation are controlled by internal signaling and endocrine signals from outside the ovary (Thomas et al., 2003).

By unknown reasons, follicles are gradually and continuously recruited and start to growth, unfortunately, the factors and hormones which stimulate or inhibit this process remain to be identified. Even if gonadotrophins receptors were identified at this stage (Bao and Garverick, 1998), this initial growth appears to be independent of pituitary gonadotrophins since the primordial follicles can still develop to the early antral stage in their absence (Awotwi et al., 1984; Gong et al., 1996). Others studies indicate that maybe several members of the growth factor beta superfamily are implicated (Erickson GF and Shimasaki S, 2001; Reddy et al., 2008; Trombly et al., 2009).

The quiescent follicle consists of an immature oocyte surrounded by a single layer of flattened granulosa cells and it is called **primordial follicle** (Gougeon, 1996) and measure approximately 30µm.

When these follicles are recruited into the growing pool, the granulosa cells grow and become cuboidal but continuous to form only one layer – **primary follicle**, this appearance is the first sign of activation and initial recruitment. The granulosa cells of

this follicle, that measure now approximately 60 µm, start to secrete mucopolysacharides around the oocyte to form the zona pellucida.

The granulosa cells proliferation forming multiple layers around the oocyte, and oocyte size increasing, are the **secondary** or **pre-antral follicle** characteristics. When there are three or more layers of granulosa cells, the surrounding stromal cells differentiate to form theca interna and theca externa, with vessels blood between it (Reynolds et al., 1992). At this stage the diameter of the follicle is 100-200 µm and granulosa cells demonstrate very high mitotic activity. Once the follicle reaches a specific size, approximately 500 µm, it stars to develop a space fluid filled within granulosa cells called antrum (Telfer and Gosden, 1987) and become acutely dependent on gonadotrophins for further growth and development (Nayudu and Osborn, 1992) – **antral follicle**. At this stage the granulosa cells differentiate to mural granulosa cells, forming a thin layer along the periphery of the follicle, and to cumulus granulosa cells that surround the oocyte, forming the **pre-ovulatory follicle**.

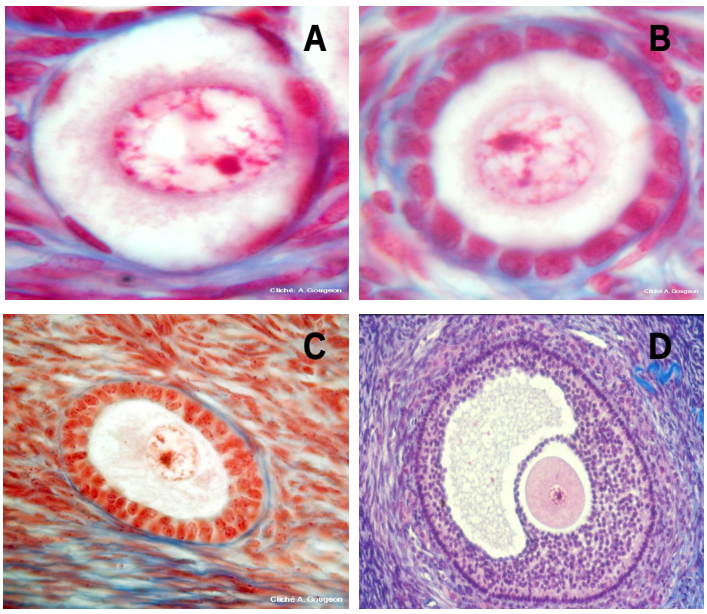

Figure 4: Human ovarian follicles at the primordial stage (**A**), the primary stage (**B**),the secondary stage (**C**) and the antral stage (**D**)
(by Gougeon in the photothèque fertilité Ferring®)

As soon as the follicle enters the growing pool the oocyte starts to grow and communicate with the surrounding follicular cells, which communicate each other, through gap junctions (Eppig, 1982). The gap junctions allow the bidirectional transfer of nutrients, metabolic precursors and signal molecules (Eppig, 1991; 1992).

This interaction is the essential factor for the oocyte and follicular development.

APOPTOSIS

Contraction followed by clumping of the chromatin, wrinkling of the oocyte nuclear membrane and dislocation of the granulosa cells (GC's) is considered signs of atresia (Baker, 1963). This atresia condition is the result of an apoptotic process that is morphologically characterized by condensation of nuclear chromatin, nucleolus disintegration and a reduction of nuclear size, simultaneously with cytoplasmic condensation and reduction of the total cell volume.

The activation of apoptotic cascade in follicles, among others, can be due to oxidative stress, insufficient tropic support and chemical messengers. All these alterations lead to the activation of endonucleases activity and disrupt prevailing ionic gradients, causing DNA damage.

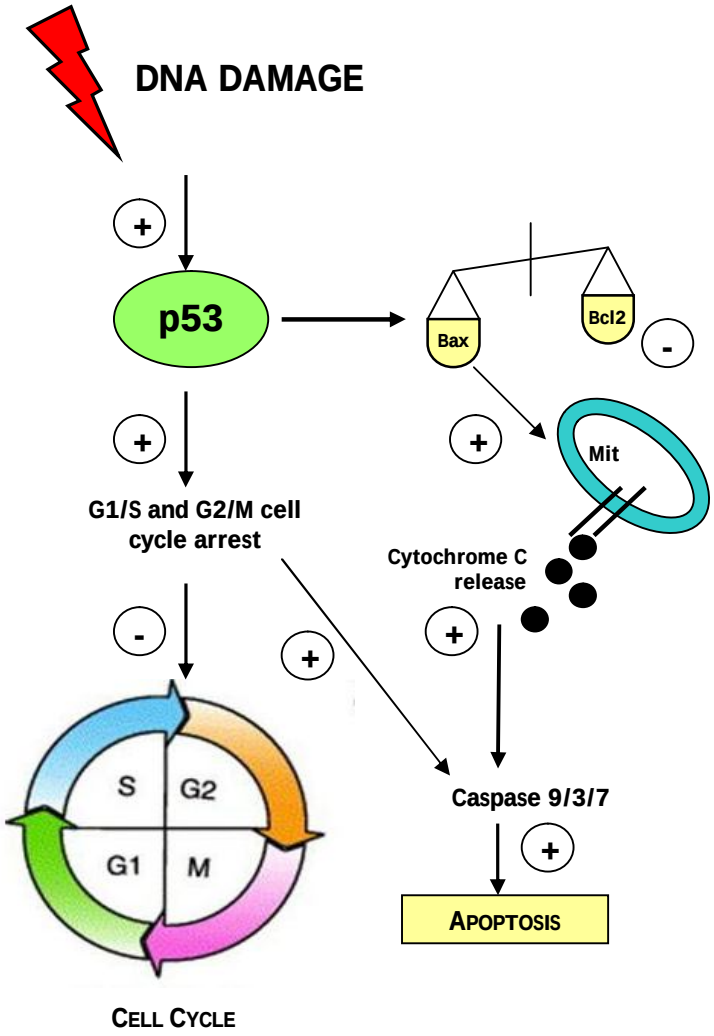

Figure 5: Diagram of p53 apoptosis pathway ("+"= stimulation; "-"= inhibition; Mit= mithocondria)
(adapted from Gillham *et al. World Journal of Surgical Oncology* 2007)

The DNA damage is a stimulus for increased p53 expression in GC's inducing the arrest of cell proliferation blocking it at G1 phase. This cell cycle arrest is reversible, and if the appropriate DNA repair is done, the p53 decrease and cell cycle continues, otherwise cell dies (Fig 5).

This process can also been prevented among others by Bcl2, that acts as antioxidant, blocking the cell death pathway, but is not associated to cell proliferation.

Briefly, the fate of GC's during development may be decided by the balance of death repressors and death inducers.

The proliferation of GC's is essential to follicle and oocyte development, being an important way to evaluate their viability. The proliferating status can be proved by the expression of Ki67 that is expressed in all phases of cell cycle excepting G0 phase.

TRANSFORMING GROWTH FACTOR BETA SUPERFAMILY

Among the many growth factors that have been identified as being important in early follicular growth several are members of the Transforming Growth Factor-ß superfamily (Erickson and Shimasaki, 2000).

The TGF-ß superfamily is a large group of 25kDa proteins, which share commons structural motifs (Chang et al., 2002). They are all synthesizes as prepropeptides with one signalpeptide, a large proregion and a smaller biologically active mature region, and exists as homo or heterodimers (Massagué, 1990; Chang et al., 2002). They are categorized into 23 distinct gene types that fall into four major groups, i.e., bone morphogenetic proteins and growth differentiation factors; activins and inhibins; TGF-ß itself and a group encompassing various divergent members as anti Mulerian hormone (AMH) (Burt, 1992; Herpin et al., 2004).

Two types of transmembrane serine/threonine kinase receptors (Type I and Type II) have been identified for these polypeptides (Massagué and Wotton, 2000; Chang et al., 2002; Miyazawa et al., 2002). When a ligand binds, the kinase of the type II receptor phosphorylate and thereby activates the kinase of the type I receptor, which in turn will activate the intracellular signaling molecules called Smad proteins (Shi and Massagué, 2003; ten Dijke and Hill, 2003; Caestecker, 2004). There are eight distinct Smad proteins known, divided in three functional classes, which are, receptor regulated Smads (Smad 1,2,3,5,8), collaborating Smads (Smad 4) and inhibitory Smads (Smad 6,7) (Shi and Massagué, 2003).

There are basically two main pathways, the TGF-ß/activin pathway where the ligand has a higher affinity for the type II receptors and the BMP pathway with ligands that have a higher affinity for the type I receptor (Shi and Massagué, 2003; Caestecker, 2004; ten Dijke and Hill, 2004). The TGF-ß/activin pathway induce the activation of Smads 2/3 while the BMP pathway activate the Smads 1/5/8, however both pathways lead to the formation of the complex regulated Smad actived/collaborating Smad (Smad

4) (Zhang and Derynck , 1999). This complex then translocates into the nucleus and activates transcription of specific genes (Zhang and Derynck, 1999). The Smads 6/7 can inhibit the Smads of the BMP pathway while the Smads of the TGFß/activin pathway only are inhibiting by the Smad 7 (Massagué, 1998; Miyazawa et al., 2002) (Fig 6).

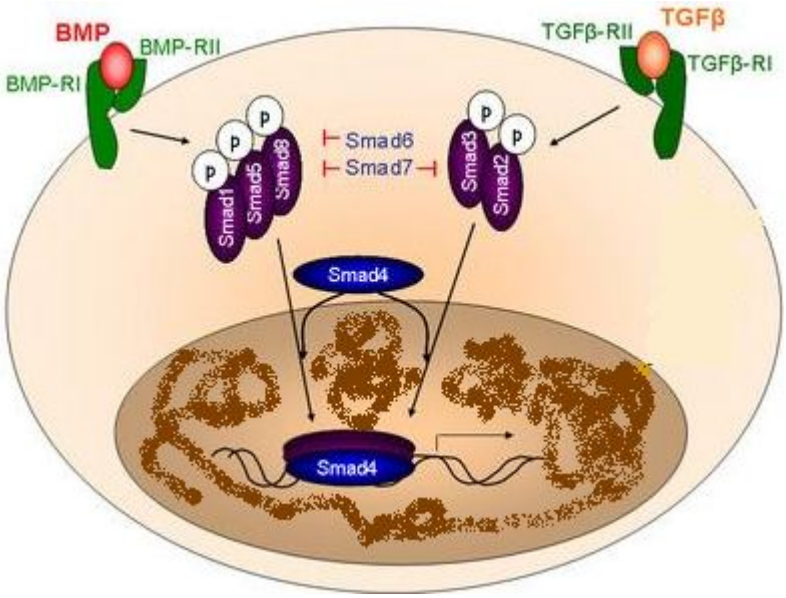

Figure 6 : The BMP and TGFß/activin signaling pathway
(adapted from http://www.rr-research.no)

GROWTH DIFFERENTIATION FACTOR-9

This growth factor was identified in 1993 (McPherron and Lee, 1993) and is expressed and secreted by the oocytes in mostly species, including humans (McGrath et al., 1995).

Mutations in the GDF-9 gene are associated with various reproductive abnormalities, among the most serious is that although the oocytes grow in their follicles, the growth of the follicular cells is retarded which results in an arrest in the follicle growth and infertility (Carabatsos et al., 1998), proving its essential role during follicular development (Dong et al., 1996).

The expression of this factor differs among the species with GDF-9 expression observed in primordial follicles of sheep, cattle, possum and hamster (Bodensteiner et

al., 1999; Eckery et al., 2002; Wang and Roy, 2004) and in primary follicles in rats, mice and humans (Laitinen et al., 1998; Jaatinen et al., 1999).

This protein function as a paracrine factor in the regulation of granulosa cell proliferation and differentiation (Elvin et al., 1999; Eppig, 2001; Gilchrist et al., 2006), and is correlated with recruitment of follicles for growth being essential for fertility (Dong et al., 1996). The higher proportion of viable human follicles in organ culture, suggest that this factor enhances the survival of the follicles (Hreinsson et al., 2002).

The role of GDF-9 in each stage of follicular development remains unclear but several studies pointed to its extreme importance for the proper development of follicle/oocyte (Dong et al., 1996; Elvin et al., 1999; Hanrahan et al, 2004; Kovanci et al., 2007), becoming crucial to understand his action mechanism .

ACTIVIN A

Activins are hetero or homo dimmers comprising two different beta subunits (A or B) with activin A being the predominant activin isoform. In the ovary, activins are produced by the GC's and are important in folliculogenesis (Findlay et al., 2002), but the exact stages of development that activin A regulated are unclear. However, it has been shown to promote follicle growth by increasing GC proliferation and enhancing antral formation (Mizunuma et al., 1999; Zhao et al., 2001).

Activin stimulated follicle *in vitro* has been demonstrated in preantral ovine follicles (Thomas et al., 2003), caprine follicles (Silva et al., 2006), rodent follicles (McGee et al., 2001) and in human follicles (Telfer et al., 2008). Furthermore, it has been observed *in vitro* that activin A stimulates the meiotic maturation of the oocytes in human, rat and rhesus monkey (Itoh et al., 1990; Sadatsuki et al., 1993; Alak et al., 1998).

There is no evidence for the role of activin A in primordial follicle activation, but his expression on this early stage is a reason to think that it can have a relevant role at this stage (Mizunuma et al., 1999), thus, further investigations will be necessary.

FERTILITY PRESERVATION

The survival rate of children and young women after treatment against malignant diseases has increased in the last decades, due to improvements in the treatment therapies (Landis et al., 1998). Nevertheless one of the side effects of these treatments (chemotherapy/radiotherapy) is the frequent loss of fertility and the onset of a premature ovarian failure (POF) ((Donnez et Bassil, 1998; Yeung et al. 1998; Smitz et Cortvrindt, 1999).

The ovaries are very sensitive to cytotoxic treatment, especially to alkylating agents that increase the risk of POF by a factor of 9 (Byrne et al., 1992).

For the abdominal radiation it has been stated that a dose of 5-20 Gy is sufficient to completely impair gonadal function (Wallace et al., 2005), moreover, the association of abdominal radiation and chemotherapy with alkylating agents could rendering patients infertile in almost 100% of the cases (Donnez et al., 2006).

Beyond cancer and autoimmune diseases treatments such as radiotherapy or chemotherapy POF can be induced by chromosomal abnormalities such Turner's syndrome (characterized by partial or total loss of one of the X chromosomes) (Sylven et al., 1993) and gene defects such as FSH receptor mutations (Aittomaki et al., 1995). Thus it is important to develop options for fertility preservation for these women.

Currently, there are three options proposed for fertility preservation: embryo cryopreservation, oocyte cryopreservation and ovarian tissue cryopreservation (Gosden, 2005; Meirrow et al., 2005). Nevertheless the choice of the most convenient strategy for preserving fertility depends on the type of the disease, the patient's age and in some countries their partner status.

EMBRYO CRYOPRESERVATION

This technique is the most widely available because she has become a routine in all Assisted Reproductive Technology (ART) centers and has proven its efficacy in terms of pregnancy and rates of delivery babies. However there are several drawbacks to the application of this technique.

First it is only applicable to post pubertal patients, who have a partner or, status law depending, are willing to use donor sperm.

Secondly, the In Vitro Fertilization (IVF) needs a cycle of hormonal stimulation for about a month that can represent an unacceptable delay to the beginning of the treatment, and at last, if the patient has a hormonal-sensitive tumour the traditional ovarian stimulation may be harmful. Recently, tamoxifen and letrozole were proposed as a safe alternative to the traditional ovarian stimulation (Oktay et al., 2005).

OOCYTE CRYOPRESERVATION

Oocyte cryopreservation is an alternative for patients with the same characteristics as those described to the embryos but who are not with a partner and/or do not wish, or can not use sperm donation. The oocyte cryopreservation can be doing at the mature or immature state but there are different advantages and inconveniences associated with each state.

Mature oocyte

The metaphase II (MII) oocyte is a large and highly specialized cell that is extremely fragile due, among other things, to the spindle apparatus that is easily damaged by intracellular ice formation (Mandelbaum et al., 2004) inducing a risk of loss of chromosomes.

Several years after the first report of a live birth after cryopreservation of matures oocytes and thawing followed by IVF (Chen et al., 1986) the success rate remained low, but the combination with intra cytoplamisc sperm injection (ICSI) slightly improved the success rate (Porcu et al., 1997; Fabbri et al., 2001) since it avoid the difficulty of the spermatozoas to penetrate the zona pellucida hardened during the freezing process.

Improvements of the freezing protocols such the concentration of cryoprotectants (Fabbri et al., 2001), the time of exposure to pre-equilibration and thawing (Yang et al., 2002) also improved the survival rate of the mature oocytes after thawing (Sonmezer and Oktay, 2004), however, the mean pregnancy rate per thawed oocyte does not exceed 1.8% (Borini et al., 2006. Levi Setti et al., 2006).

Recently the ultrarapid freezing with vitrification offer advantages over conventional slow cooling protocols by improving post-thawing survival rates avoiding the formation of intracellular ice. A study demonstrates a rate of 5.1% live birth per warmed oocyte after vitrification (Kim et al., 2009).

Immature oocyte

This oocytes at the GV stage, survive better to the cryopreservation procedure (Boiso et al., 2002) but there maturation *in vitro* is still suboptimal with only one live birth reported (Tucker et al., 1998).

OVARIAN TISSUE CRYOPRESERVATION

This is the only technique that can be proposed to children and patients who need immediate chemotherapy (Poirot et al., 2007).

Cryopreservation of ovarian tissue is considered a promising method for preservation fertility due to the large number of immature oocytes existing in the ovarian cortex.

After the first studies with animals in the 1950s (Parkes et al., 1953) and offspring obtaining in mice after transplantation (Parrott, 1960) the revival of the interest in cryopreservation of follicles in ovarian cortical tissue was in the 1990s.

The first human ovarian tissue cryopreservation was in 1996 (Hovatta et al., 1996; Newton et al., 1996) using different cryoprotectants to prevent the ice crystals formation in the cells. The cryoprotective agents commonly used are dimethyl sulfoxide (DMSO) and propanediol (PrOH) or ethylene glycol (EG) at 1.5M with sucrose 0.1M (Hovatta et al., 1996; Newton et al., 1996; Gook et al., 1999). No significant differences have been observed between DMSO, PrOH in terms of follicle survival (Newton et al., 1996).

The follicles are cryopreserved embedded in the ovarian tissue using a controlled-rate cooling followed by storage in liquid nitrogen at -196°C. The tissue is previously divided into small fragments to facilitate the permeation of the cryoprotectant.

Studies concerning follicular viability report that at least 70% of the follicles can be expected to survive the process of cryopreservation and thawing (Hovatta et al., 1996; Oktay et al., 1997; Gook et al., 1999; Cortvrindt and Smitz, 2001; Nisolle et al., 2000).

The quiescent primordial follicles are the most able to survive due to their size and low number of granulosa cells being easily equilibrate with the cryoprotectant. Those follicles have a low metabolic rate, the zona pellucida is not yet formed and their oocyte is arrested in the prophase of the first meiotic division (Shaw et al., 2000).

Recently, vitrification was proposed as an alternative procedure to slow cryopreservation. This technique uses a high concentration of cryoprotective agents and an immediate freezing, transforming the aqueous parts of the cells in a solid amorphous vitreous stage and avoids the formation of ice crystals.

The handicap of those options is that the follicles surviving the cryopreservation process contain immature oocytes. These oocytes can not be immediately fertilized, they need further maturation. This maturation can be achieved *in vivo* by transplantation or *in vitro* by culture of the ovarian tissue.

FERTILITY RESTORATION

OVARIAN TISSUE TRANSPLANTATION

This is to date the most viable utilization of the ovarian cryopreserved tissue allowing reestablishing the hormonal balance and thereby delay menopause and also restore fertility.

The transplantation can be done in its original site (orthotopic), in a well vascularized site in the woman body (heterotopic) and to another species (xenotransplantation).

The viability of this technique was demonstrated with offspring obtained in animals and in humans.

Heterotopic transplantation

This technique involves the transplantation of the tissue to another part of the body as the forearm (Oktay et al., 2001) or the abdomen wall (Oktay et al., 2004). With these options the women resumed normal menstrual cycle avoiding the hormone replacement therapy and oocytes have been obtained. Nevertheless, this technique didn't result in evolutive pregnancy but it was shown for the first time that oocytes and embryos could be obtained from cryopreserved/thawed ovarian tissue, in human (Oktay et al., 2004).

Orthotopic transplantation

In this case the tissue is implanted at its original site. The first human transplantation was reported by Oktay and Karlikaya (2000), who obtained ovarian function after sixteen weeks and even an ovulation after stimulation. This study proved that the cryopreservation protocol was not totally harmful for human ovary.

The first live human birth was obtained in 2004 (Donnez et al., 2004) in a patient without previous treatment before the cryopreservation. This, open the discussion of the really nature of the pregnancy; "*oocyte from the remaining ovary or from the transplantation?*". But the next year it was published that a health child was born after

orthotopic transplantation of slices of ovary cryopreserved after a cancer treatment (Meirow et al., 2005).

Since twenty four transplantations were reported and eight pregnancies obtain with six babies born.

Table 1 : Autotransplantations of cryopreserved human ovarian tissue (adapted from Poirot et al., 2008)

REFERENCE	AGE BEFORE FREEZING	GRAFT TYPE	OUTCOME
Oktay K (2000)	29	Orthotopic	FD after stimulation
Radford J (2001)	36	Orthotopic	Ovarian function
Callejo J (2001)	47	Heterotopic	Endocrine ovarian function
Oktay K (2004)	30	Heterotopic	Embryo
Tryde Schmidt KL (2004)	32	Orthotopic	MII oocyte
Donnez J (2004)	25	Orthotopic	**Live birth**
Kim S (2004)	37	Orthotopic	Ovarian function
Meirow D (2005)	28	Orthotopic	**Live birth**
Schmidt KL (2005)	28	Orthotopic & Heterotopic	Ovarian function
	25	Orthotopic & Heterotopic	Embryo
	32	Orthotopic	Embryo
Wolner-Hanssen P (2005)	30	Heterotopic	FD after stimulation
Donnez J (2006)	21	Orthotopic	Ovarian function
Rosendhal M (2006)	28	Orthotopic & Heterotopic	Pregnancy
Demeestere I (2006)	24	Orthotopic & Heterotopic	Pregnancy
Demeestere I (2007)	24	Orthotopic & Heterotopic	**Live birth**
Donnez J (2008)	22	Orthotopic	Oocytes
	28		Cycles
	22		Cycles
Andersen CY (2008)	26	Orthotopic	**Live birth**
	27	Orthotopic	**Live birth**
	36	Orthotopic	Ovarian function
	25	Orthotopic & Heterotopic	
Silber SJ (2008)		Orthotopic	**Live birth**

FD – follicular development ; MII oocyte – oocyte in metaphase II

Xenotransplantation

This technique is commonly done for viability studies and not as a clinical option. The studies, using immunodeficient mice, show good recovery of follicles and follicular growth to late and antral stages (Newton et al., 1996; Gook et al., 2001; Kim et al.,

2002). Ovulation and the formation of corpus luteum has also been observed with this technique (Gook et al., 2003).

OVARIAN TISSUE CULTURE

For some diseases, the transplantation of the thawed fragments of ovarian tissue represents a high risk of reintroducing malignant cells (Shaw et al., 1996; Gosden et al., 1997; Aubard et al., 1999). The maturation of the follicles *in vitro* could avoid this risk.

The development of primordial follicles to an antral stage and consequent oocyte maturation is a complex procedure and until now, completely successful only in mice with live offspring born (Eppig and O'Brien, 1996; O'Brien et al., 2003).

In Humans the *in vitro* development, with or without cryopreservation of ovarian tissue was reported but with fewer results than those observed in rodents, but still hopeful for further investigation. The complete follicular development was not yet report, but the various stage of development obtained separately shows that human folliculogenesis *in vitro* is possible. The long time required for follicle development (Gougeon et al., 1987) and the dense ovarian stroma make this culture more challenging (Hovatta et al., 1997; Lass et al., 1997).

Under the last ten years multiple studies regarding culture of fresh and cryopreserved ovarian tissue tried to discover and establish the perfect conditions to lead to complete and successful *in vitro* follicle development.

In Humans several works described ovarian follicles growth from a state to the following state. Some develop techniques using preantral follicles isolated mechanically (Abir et al., 1997) or by enzymatic digestion (Roy and Tracy, 1993) that after culture observed antral cavity formation. However those achievements were hardly reproduce with primordial and primary follicles (Abir et al., 1999; Hovatta et al., 1999), showing that for this type of follicles the culture within ovarian tissue is recommended (Hovatta et al., 1997, 1999; Hreinsson et al., 2002; Scott et al:, 2004).

Recently a team reported a two-step culture (Telfer et al., 2008), trying to reproduce the good achievements obtained with this technique in mice (Eppig and O'Brien, 1996; O'Brien et al., 2003). In this protocol the small follicles, were in a first time cultured within a thin slice of ovarian tissue until they reach the secondary state. From there, the secondary follicles obtained were mechanically isolated and cultured until antral cavity formation was observed (Telfer et al., 2008). The particularity of this study is the short time need to all this process (10 days), and the largest diameter

obtained for follicles cultured in a medium with activin A than in follicles cultured without this additional factor.

The addition of different growth factors and/or hormones in the culture medium as been study trying to improve the development and survival of the follicles *in vitro* (Wright et al., 1999; Louhio et al., 2000; Hreinsson et al., 2002; Telfer et al., 2008).

Among the factors that are pointed to regulate the initiation and progression of primordial development, Kit Lingand (KL) and b-fibroblast growth factor (bFGF) seem to be essential for their progression to primary follicles (Parrot and Skinner, 1999; Nilsson and al., 2004) while GDF-9 are fundamental to their development into the secondary stage (Hreinsson et al., 2002). Insulin, insulin like growth factor I (IGFI) and IGFII act as trophic factors for follicles and simultaneously stimulate follicular growth (Fabbri et al., 2006; Louhio et al., 2000) and gonadotropins, FSH and LH are essential for progression from the preantral to the antral follicle stage (Wrigth et al., 1999).

The utilization of an extracellular matrix in the culture of isolated follicles to mimics the ovarian conditions were described successful (Roy and Treacy, 1993; Abir et al., 1999, 2001; Woodruff and Shea, 2007) but Telfer shows that it is not essential to obtained follicle growth (Telfer et al., 2008).

The presence or absence of serum in the culture medium is equally discussed with follicular growth observed in the two cases.

Although all progress made in the culture of human follicles, this reality and clinical application is still far from the optimum conditions. Further experimentation is needed to fully understand the entire continuum of follicle development, optimize timings of exposure to key factors and to evaluate the viability of oocytes obtained to be fertilized and develop into normal embryo.

AIMS OF OUR STUDY

While most of previously works were realized on fresh ovarian tissue, the clinical reality is the future utilization of cryopreserved ovarian tissue, being important to realize and evaluate culture of cryopreserved ovarian tissue.

The aim of our study was to analyze the effect of two growth factors added into medium, evaluating 4 parameters of the follicle growth.

To accomplish it we realized:

- Culture of human cryopreserved tissue simultaneously in three different culture media
- Counting and morphological analyze of follicles
- Observation of eventually changes in the morphological aspect of the stromal and vascular tissue into the ovarian fragments, along the culture
- Immunohistochemical analyze of the follicles, stromal and vascular tissue using two markers involved in apoptosis (p53 and bcl2) and one marker of cell proliferation (Ki67)

MATERIALS AND METHODS

OVARIAN TISSUE

Human ovarian fragments frozen for fertility preservation of one 15 years old patient were used.

The patient and her parents, before cryopreservation, signed an agreement for postmortem utilization, for research, of the frozen tissue.

The isolated ovarian cortex has been cryopreserved as little fragments of about 0,5 cm^3 following the slow freezing method described by Gosden (Gosden et al., 1994) using DMSO and sucrose as cryoprotectants.

All manipulation of the human ovarian tissue was done with sterile material and under sterile conditions.

OVARIAN TISSUE THAWING

The ovarian fragments were thawed one by one as follow.

The cryovial was pull out of the liquid nitrogen and placed for a few seconds at room temperature followed by five minutes in the incubator at 37ºC, till complete defrost of the cryopreservation medium.

After this process the fragment was washed, at room temperature, successively in 4 different solutions containing a decreasing concentration of DMSO (2M; 1,5M; 1M; 0M). The thawing solutions were composed by Leibovitz medium (Eurobio®, Courtaboeuf, France), DMSO (Sigma Aldrich®, St Quentin Fallavier, France), fetal calf serum (FCS) (Eurobio®) and 0.2M sucrose (Sigma Aldrich®).

PREPARATION OF CORTICAL TISSUE FOR CULTURE

After thawing the fragments were placed in a prewarmed Leibovitz medium (Eurobio®) supplemented with sodium pyruvate (2mM,), bovine serum albumin, BSA, (Fraction V, 3mg/mL), ascorbic acid (50 µg/mL,) and stabilized solution of L-Glutamin,

penicillin G and streptomycin (2mM, 75µg/mL, 50 µg/mL, respectively) all purchased from Sigma-Aldrich®.

In this medium the fragments were cut into smaller pieces (~ 0,5 mm^3) with a scalpel under a stereo microscope and pulled mechanically with 25 gauges needles (Sherwood médical®, Evry, France) to flatten out the tissue and to minimize the underlying stromal tissue.

DIFFERENT CULTURE MEDIA COMPOSITION

For this work three different culture media were used.

The medium used as control group was composed of McCoy's 5a medium (Sigma-Aldrich®) supplemented with HEPES (20mM, Eurobio®), BSA (0,1%), stabilized solution of L-Glutamin, penicillin G and streptomycin (3mM; 0,1mg/mL; 0,1g/mL, respectively), transferrin (2,5µg/mL), selenium (4ng/mL), insulin (10ng/mL) and ascorbic acid (50µg/mL), all purchased in Sigma Aldrich®.

The two other media were composed by the control medium which has been supplemented with 200ng/mL of GDF-9(CliniSciences®, Montrouge, France) or with 100ng/mL of activin A (R&D Systems®, Lille, France).

The different culture medium gave name for the different groups, so there were formed 3 groups:

- **C group** – control culture medium
- **G group** – culture medium with GDF9
- **A group** – culture medium with activin A

CORTICAL STRIP CULTURE

This work was accomplished in four different time cultures.

The cortical strips were cultured individually in 24-well culture plates (Costar®,), containing 300 µL of the respectively culture medium, using a culture plate for each medium.

The three groups are incubated (at the same time) at 37°C in humidified air with 5% CO_2. Half of the medium, of each well, was changed every 2 days. This pulled medium was stored at -20°C for further hormonal dosing.

Along the culture the strips were observed at the inverted light microscope, to verify the presence of developing follicles.

A total of 247 cortical strips were cultured (82 in the control group; 82 in the GDF-9 group and 83 in the activin A group).

HISTOLOGICAL ANALYSIS

Before placing the cortical strips in the different culture media, 4-8 strips (from each fragment used), were fixed in a 10% formaldehyde solution overnight.

At day 5, 10 and 14, of culture, eight strips of each group were fixed in a 10% formaldehyde solution overnight.

At each time of fixation the strips of the same medium were fixed together.

After fixation with 10% formaldehyde the samples were dehydrated in ethanol, using increase purity baths (70%, 90% and 100%). The absolute alcohol was then replaced by xylene and followed by paraffin inclusion of the samples.

The blocks were sectioned manually at the microtome (3 or 4µm), mounted on charged slides and left to dry for at least one night at 37ºC. Each slide contained 2 or 3 serial sections.

MORPHOLOGICAL ANALYSIS

For this analysis the serial sections obtained were staining with haematoxylin, eosine and safranin (HES).

Every section in the slide was examined, by screening, to make the counting, morphological classification and evaluation of the follicles and intuitive evaluation of the stromal and vascular tissue.

Follicle Counting and Classification

The global counting of follicles includes all follicles observed, but for the classification only follicles with visible nucleus were counted, for evaluation of all parameters of the oocyte. The sections with visible nucleolus were chosen in preference.

The follicles were classified as follow (Table 2).

Table 2: Characteristics observed and follicle stage associated

FOLLICULAR STAGE	CHARACTERISTICS
Primordial	Oocyte surrounded by one layer of flattened GCs
Early primary	Oocyte surrounded by one layer of flattened and cuboidal GCs
Primary	Oocyte surrounded by one layer of cuboidal GCs
Secondary	Oocyte surrounded by two or more layers of cuboidal GCs

Others evaluation criteria

The morphological evaluation of follicles was made only in the classified follicles by **counting the GC's**, observing **chromatin distribution** (diffuse/dense) and **oocyte cytoplasm configuration** (normal/vacuolar/retracted).

IMMUNOHISTOCHEMICAL EVALUATION

The immunohistochemical staining was performed by an automated immunohistochemical processor (Benchmark XT, Ventana®, Illkirch, France) using HRP (horsedarish peroxidase) as reactive and DAB (diaminobenzidine tetrahydrochloride). The counterstaining was realized with haematoxylin II, all reagents were purchased in Ventana®.

The primary antibodies used were monoclonal mouse anti-Human Ki67 (clone MIB-1) 1/50 diluted, p53 (clone DO-7) 1/25 diluted and Bcl2 (clone 124) 1/50 diluted, all obtained in Dako®, Trappes, France.

The immunostaining was considered positive when the cells were observed with brown coloration, for all antibodies, and negative when cells observed are blue.

Immunohistochemical evaluation of follicle was realized counting the number of GC's positively marked versus GC's negatively marked.

Stroma and vascular cells marked positively were counted.

STATISTICAL ANALYSIS

Quantitative values were treated with ANOVA with effect medium/day of culture.

Binary variable were treated with logistic model with effect medium/day and interceptions within it.

Ordinal variables were treated with logistic model ordinal with 3 or 2 modalities. For all tests the significatif value considered was $p<0.05$.

RESULTS

MORPHOLOGICAL ANALYSIS

Follicle Counting and Classification

For this analyze 46 slides with each 2 or 3 serial sections of 8 grouped strips were observed.

The counting of all follicles show a significant ($p < 0.05$) decrease in the number of follicles observed since the day 10 of culture for all culture media (Fig 7).

Even if the different culture media did not present a significant difference it was observed a higher number of follicles in the G group. At the end of the culture this was the group with a higher number of follicles and a lower proportion of degenerate follicles.

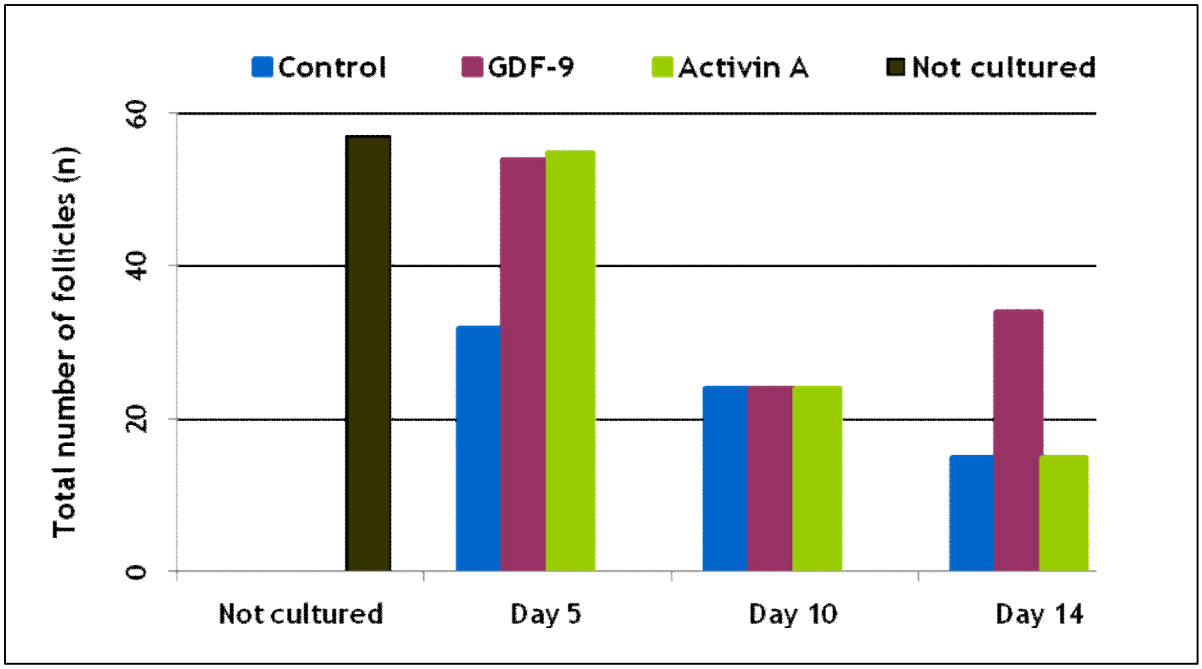

Figure 7: Total number (n) of follicles counted for each culture media at the different time of culture

Throughout the culture three types of follicles, primordial, early primary and primary were observed (Fig 8).

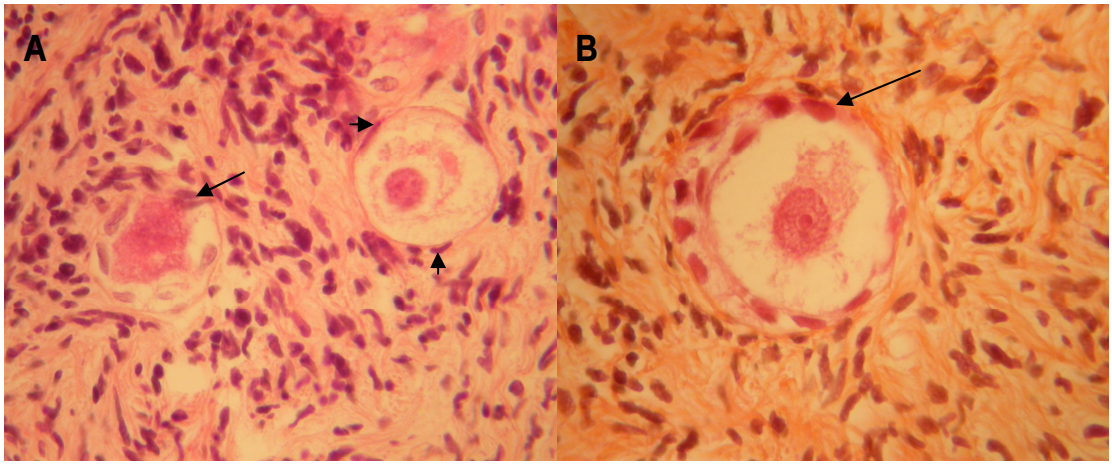

Figure 8: Microscopy visualization of a histological section with HES staining of primordial follicle and primary follicle (A) and early primary follicles (B),in ovarian cortical strip.
↑Indicates the cuboidal cells; ↑ Indicates the flattened cells

The distribution of the different types of follicles varies in the three culture media and throughout the time but without significant difference and no evidence of follicle growth (Fig 9).

- Histological sections of the cortical strips (n=28) fixed after thawing (**not cultured**) showed 57 follicles, distributed in 12 strips, where follicles were visualized. According to our counting method, from these follicles, only 31 were classifiable in the different stages (19 primordial, 3 early primary and 9 primary).

- **At the 5th day of culture**, in the obtained sections, an increase of the proportion of early primary and primary stage were registered, especially in the GDF-9 group (G) where 65% of the classifiable follicles were at the early primary stage. The activin A group (A) present the higher proportion of primary follicles (42% vs 12% for G and 33% for the control group (C) and 30% for not cultured). The primordial stage of development appears, at this time, in low number in the three culture media.

❍ **At the 10th day of culture** only 13 of the observed follicles were classifiable, and were equally distributed in the three groups. The observation of the histological sections showed an increased of the primary follicles for de G group and a decrease of the proportion of early primary follicles in the same day, compared with the 5th day of culture. For the others two culture media, the number of the three different types of follicles (primordial, early primary and primary) observed decreased comparing to the 5th Day but a similar proportional expression was registered.

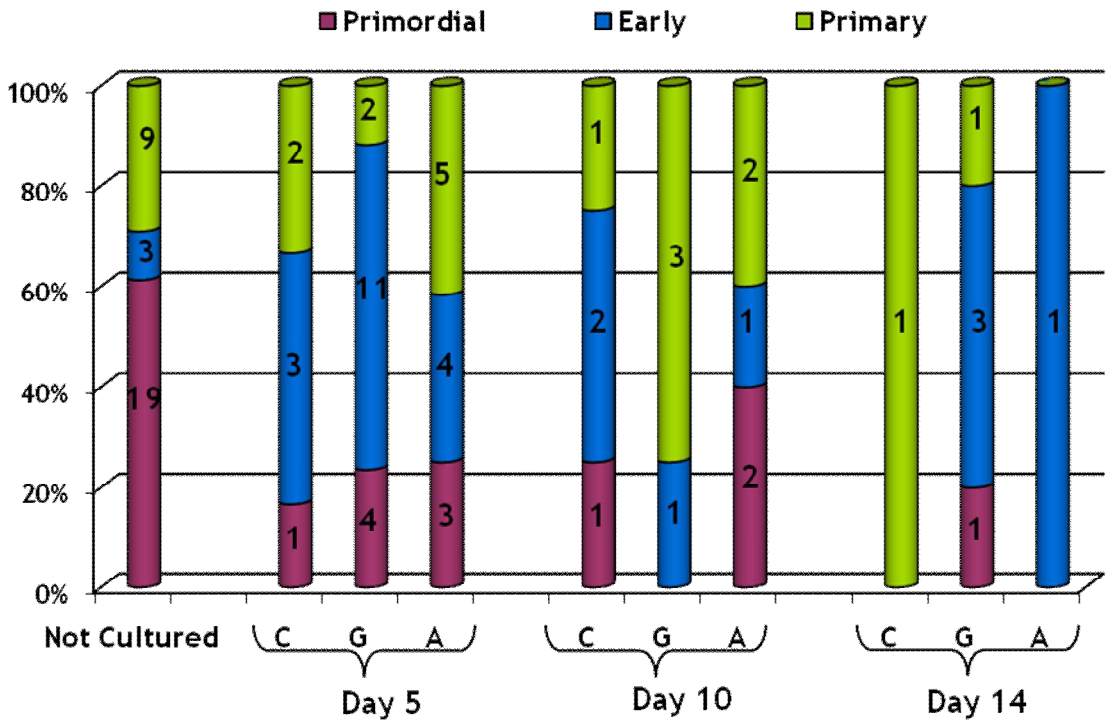

Figure 9: Distribution of the different types of follicles observed during the culture, for the three culture media, and for the not cultured ovarian fragments

❍ **After 14 days of culture** only a few number of follicles were classified (7), with the presence of one follicle for the C and the A groups (primary and early respectively). The five remainder follicles were observed in the G group and were distributed among the 3 types of follicles with proportions that are close to those found at the 5th Day.

In all sections analyzed no secondary follicles were seen.

Others evaluation criteria

The average of granulosa cells that surrounded the oocyte forming the follicle presents small variations in each medium during the culture (Table 3). However in the 10th day of culture the G group shows a mean of 11 GCs for each follicle instead of the 7 observed at the 5th day of culture. In the opposite the activin A group at the end of the culture only presented 3 GCs.

Table 3: Average number of granulosa cells per follicle, calculated, by the histological counting of granulosa cells surrounding classifiable follicles, for each culture media at the different times of culture, and for not cultured follicles

		5th Day			10th Day			14th Day		
	Not Cultured	C	G	A	C	G	A	C	G	A
GC/foll *(n)*	7	5	7	5	6	11	5	6	7	3

The chromatin configuration and the cytoplasm aspect are morphological markers of the oocyte quality.

The variations observed throughout the culture are shown in the table 4. Highlighting that, the normal aspect of the cytoplasm was mostly observed at the not cultured follicles, or in the follicles that were cultured only for 5 days. However, only at the end of the culture we register a higher proportion of retracted cytoplasm. In generality, we observe that in this culture, the oocyte presents a vacuolar cytoplasm.

Table 4: Proportion of the different aspects of the cytoplasm in the oocytes observed, for each culture media and at different times of culture, and for not cultured follicles

		5th Day			10th Day			14th Day		
	Not Cultured	C	G	A	C	G	A	C	G	A
Normal *(%)*	16,1	0	17,6	16,7	0	0	0	0	0	0
Vacuolar *(%)*	61,3	66,7	58,8	66,7	100	75,0	40,0	100	40,0	0
Retracted *(%)*	22,6	33,3	23,5	16,6	0	25,0	60,0	0	60,0	100

The proportion of oocyte with dense or diffuse chromatin didn't differ in the not cultured oocyte, however after 5 days of culture it was observed a higher proportion of oocyte with diffuse configuration of chromatin. This behavior was inverted at the 10th day of culture for the C and G groups that have a higher proportion of oocytes with dense chromatin. At the end of the culture only the G group shows oocytes with dense chromatin, however the observation for the C and A groups was made on a single oocyte for each group (Table 5).

Table 5: Proportion of the follicles with a diffuse or a dense chromatin configuration, for the not cultured follicles and those cultured in the different culture media, at different time of the culture

		5th Day			10th Day			14th Day		
	Not Cultured	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
Diffuse *(%)*	48,3	100	82,3	91,2	25,0	25,0	75,0	100	40,0	100
Dense *(%)*	51,7	0	17,7	8,8	75,0	75,0	25,0	0	60,0	0

DEGENERATED/ATRETIC FOLLICLES

For calculate the proportion of the degenerating follicles we used as total follicle population the classifiable, no classifiable and degenerating follicles (Fig 10).

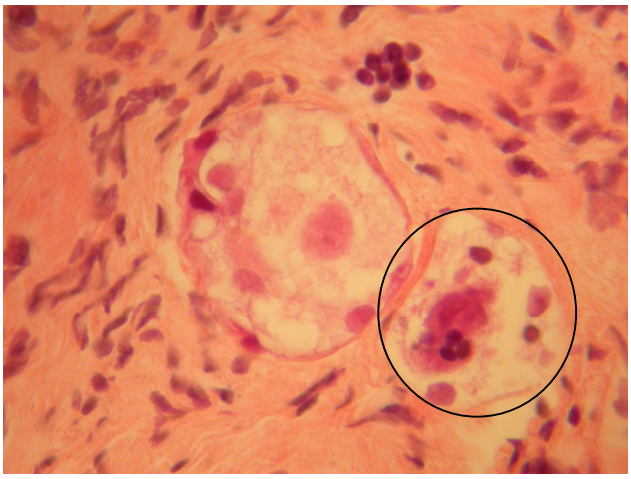

Figure 10: HES histological section of a primary and a degenerating follicle of not cultured strips; original magnification X 400

After 5 days of culture the three groups present a higher proportion of degenerated follicles when compared with the not cultured follicles (Fig 11). Along the culture time the difference between the groups increase. After 10 days of culture the A group present the higher proportion of degenerating follicles (58%) and the G group the lower proportion (25%) ($p<0,001$). The proportion of degenerated follicles observed was higher at the end of the culture, for the three groups (Fig 11).

This is the only parameter where a real difference was observed between the different culture media and the time of culture.

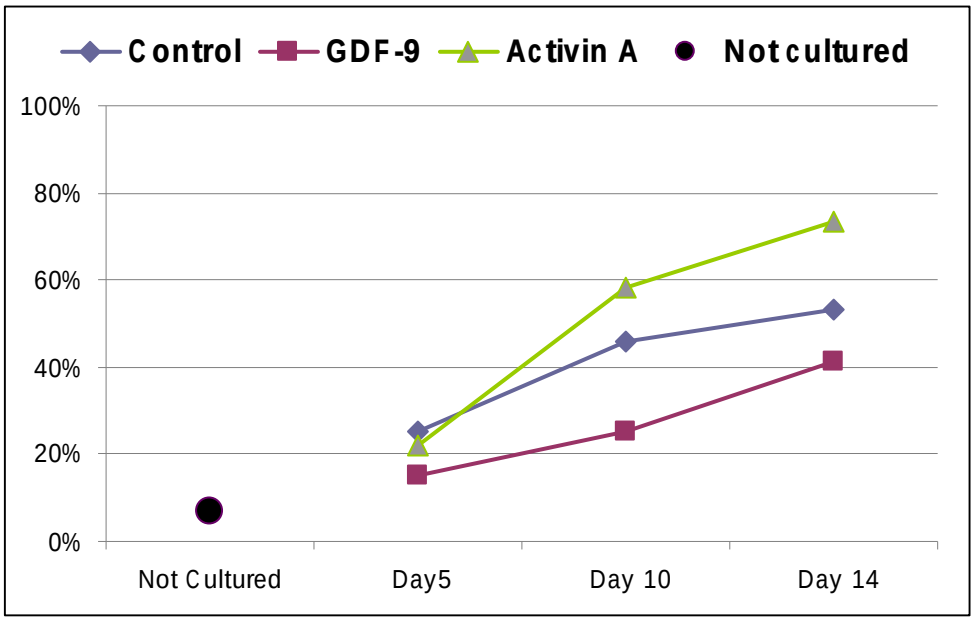

Figure 11 : Proportion of degenerating follicles counted in the different culture media, at different times of culture and in not cultured strips

The deterioration of the tissue observed during the culture was confirmed by the histological analysis, with a badly quality of stroma and vessels at the 14th day of culture (Fig 12), and a low quantity of follicles visualized at this time of culture.

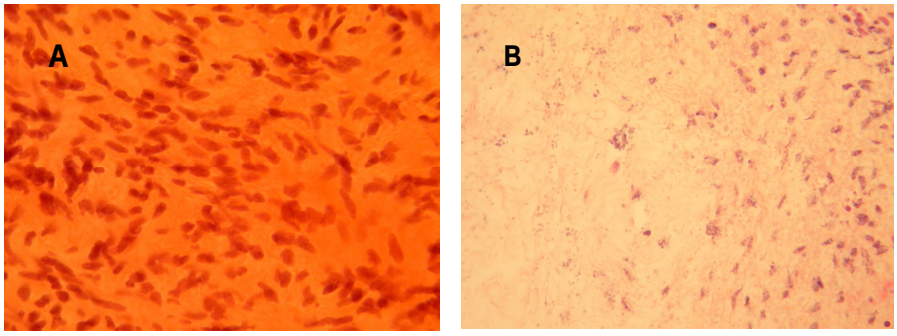

Figure 12 : HES sections of cultured stroma after 5 days of culture (A) and 14 days of culture

IMMUNOHISTOCHEMICAL ANALYSIS

For the immunohistochemicals markers the type of follicle was not a criterion for counting GCs, thus all GCs of all follicles were counted. Thus, follicles with or without a nucleus were counted. Nevertheless and as the previously parameters the screening observation was made being attention to not count twice the same follicle.

Apoptotic markers (P53 and Bcl2)

The number of follicles counted in those sections show, as the HES slides, the diminution of the number of follicles along the culture and consequently the number or GCs.

The number of granulosa cells positively marked with p53 (Fig 13) observed was changing for the three media and at the different times of culture (Table 6) without a real tendency of the proportion of positive cells. In the not cultured strips none positively marked cells was visualized.

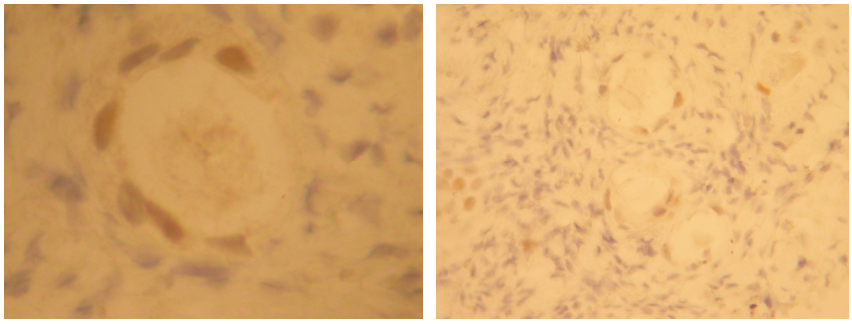

Figure 13: Cells staining brown are positively marked, cells staining blue are negatively marked to the p53

At the end of the culture we observe only one follicle in the A group and the C group, with all GCs marked positively for the first and none for the C group.

Except the 14th day of culture the group with less cells positively marked was the G group. It is also in this group that we counted more cells and more follicles.

Table 6: Follicle counting and proportions of positive granulosa cells marked for p53 antigene in a total of GCs in the three culture media, during the culture and in the not cultured strips

		5th Day			10th Day			14th Day		
	Not Cultured	C	G	A	C	G	A	C	G	A
+ GCs *(%)*	0	50	48,5	65,7	58,8	26,7	47,8	0	40,1	100
Total GCs *(n)*	91	64	194	102	17	90	23	5	22	2
Follicles *(n)*	14	11	34	19	3	13	7	1	5	1

The cells of the stromal and vascular tissue positively marked were also register, but no counting of not marked cell was done. The frequency of cells of those tissue marked were low and no depended medium and time of culture was verified. We just observe that generally for the three media and at all time of culture the vessels have more cells positively marked than in the stromal tissue.

For the Bcl2 we didn't observed granulosa cells positively marked. The only positive cells marked were cells of the vessels, but in a very low number.

Proliferation marker (Ki67)

In the sections obtained a low number of cells positively marked (Fig 14) were found for all the culture media with the trend of decreasing number of follicles depending on the time of culture, previously verified in the apoptotic markers and HES staining.

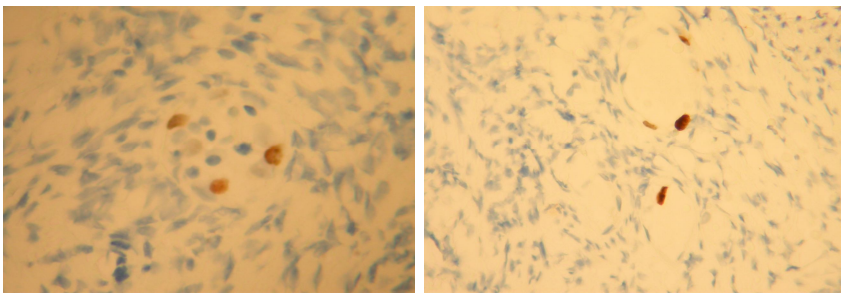

Figure 14: GCs marked for Ki67, cells staining brown are positive and cells staining blue are negative

Excepting the 10th of culture the G and C groups have similar proportions of positive cell markers and in the total counted follicles (n=37 vs n=30, respectively. In opposition the A group represents the lower proportion of positive GCs, of total GCs and follicles (Table 7).

Table 7: Follicle counting and proportions of positive granulosa cells marked for Ki67 antigen in a total of GCs in the three culture media, during the culture and in the not cultured strips

		5th Day			10th Day			14th Day		
	Not Cultured	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
+ GCs *(%)*	2,5	20,6	21,7	13,1	11,1	22,6	5,9	16,7	14,9	0
Total GCs *(n)*	119	126	180	99	63	31	17	18	47	5
Follicles *(n)*	17	16	27	14	10	5	5	4	5	1

The cells of the stromal and vascular tissue positively marked were also register, but no counting of not marked cell was done. The frequency of cells of those tissue marked were low but seems to be time depended, with more positively cells marked at the 5th day of culture for all groups, for both type of tissue.

Except the G group, where we verified a high number of cells of the stroma positively marked after 10 days of culture, in the 10th and 14th day of culture a high decrease of the cells marked was observed.

DISCUSSION

The survival rate of young patients after chemotherapy and/or radiotherapy has improved in the last decades. Thus, the gonadotoxic effect of these treatments became a relevant issue, namely the premature ovarian failure, being important to preserve fertility of those patients.

The cryopreservation of the ovarian tissue is a fertility preservation method and is the only one that can be proposed to prepubertal girls (Poirot et al., 2007). Nevertheless, in the ovary the oocytes are immature being necessary to induce their maturation for further fertilization.

The transplantation of the cryopreserved tissue is the currently technique to maturate those oocytes. However, with transplantation and for some diseases there is a great risk of reintroduction of malignant cells, thus the maturation *in vitro* is the goal.

For the last 20 years multiply studies concerning the *in vitro* growth of follicles were executed using animal models and more recently using ovarian tissue, mostly with fresh ovarian tissue.

Conversely the clinical application will be with cryopreserved tissue, that's why we chose to only use cryopreserved tissue for our study.

Recently, a study report the efficacy of a two steps culture of ovarian tissue to obtain small antral follicles in a short time (10 days) (Telfer et al., 2008). In this study developing follicles were obtained in a serum free medium and without matrix support, after a culture of cortical tissue, during 6 days, followed by the isolation and culture of preantral follicles. The second step of this culture (culture of isolated follicles), was a comparative study of the effect of activin A in human folliculogenesis, since the same author has demonstrated that this protein, produced in the granulosa cells, have a proliferating effect in the preantral bovine follicles (Thomas et al., 2003).

Since we didn't find studies revealing an inhibitory effect of activin A in the culture of ovarian tissue, and that the precise stage of action of activin A is controversial (Zhao et al., 2001) we decided to used this protein since the beginning of the culture.

Indeed we decided to develop a work comparing three culture media, using activin A and GDF-9. The medium used previously by Telfer (Telfer et al., 2008) to perform the culture of cortical strips were used as control, and to this medium we added activin A or GDF-9. The choice of GDF-9 was due to a positive effect on the

growth and development of secondary follicles, in a culture of fresh human tissue previously demonstrated (Hreinsson et al., 2002).

With this study we wanted to evaluate differences in parameters of follicular growth and development for the three culture media and thus the effects of GDF9 and activin A in the ovarian follicles developing *in vitro*.

We decided to extend the culture to 14 days instead of the 6 days as Telfer, since the observation of the cortical strips in culture, didn't allowed to detect the presence of secondary follicles for isolation and in this way we could evaluate the possible developing of the follicles in the tissue, *in vitro*.

In this work the proportion of the different types of follicles seems to be independent of the culture media. Nevertheless the diminished number of primordial follicles counted, for the G group, the increase of early primary follicles after 5 days of culture and the counting of more primary follicles in the 10th day of culture suggest that during those 10 days of culture with this culture medium a follicular development occur. Hreinsoon showed a higher proportion of secondary follicles after 7 days of fresh ovarian tissue culture in a medium with GDF-9, but also with human serum albumin (HSA).

In the 5th day of culture the presence of follicles, in the G group, was confirmed by visual observation of follicles in the cortical strips (Fig 14).

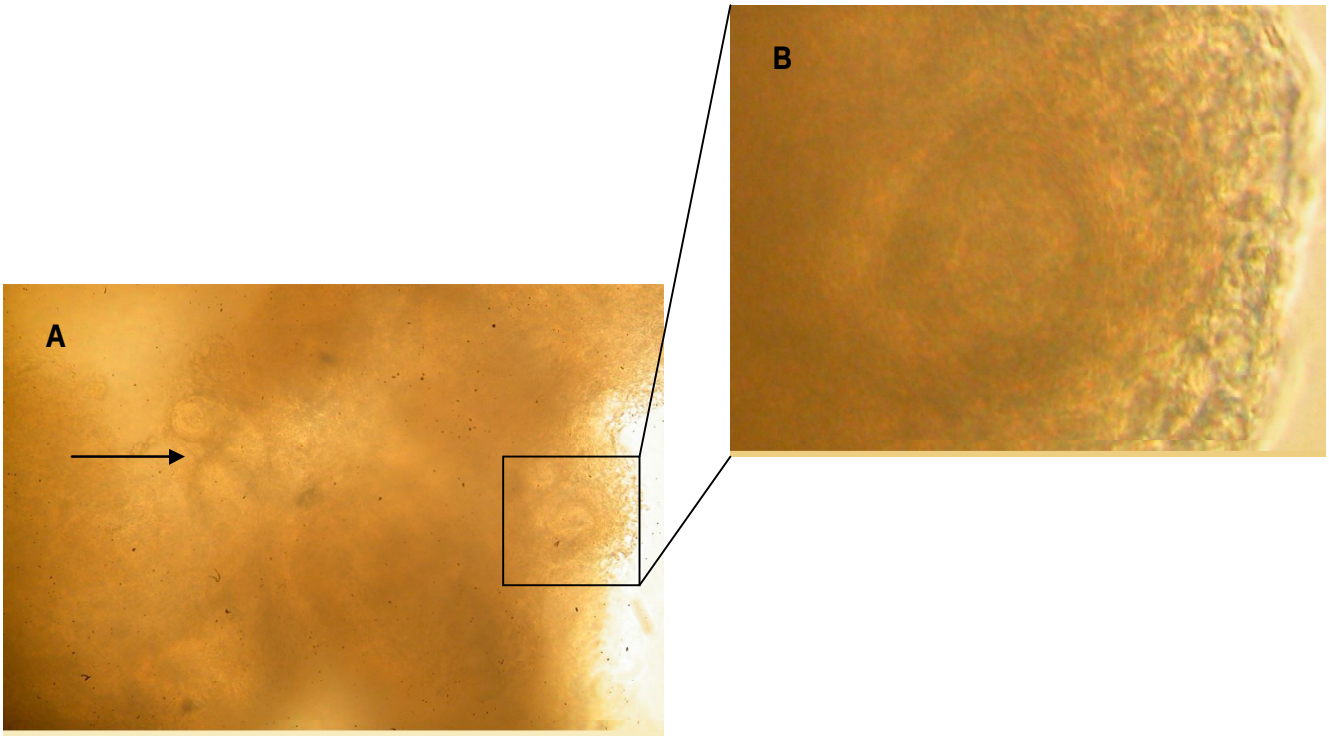

Figure 15 : General view, with the inverted microscope (200X) of follicles in a cortical strip, in the GDF-9 medium, at the 5th Day (A) and of one follicle in the peripheral zone (B)

The higher number of follicles and GCs counted, the lower proportion of degenerating follicles (15% at day 5, 25% for 10th day and 41% at the end of the culture) in the G group, corroborates the previously results that indicate this growth factor as a survival factor (Hreinsson et al., 2002).

In the culture of preantral follicles the activin A was also indicated as a survival factor (Telfer et al., 2008), that is not verified in the present study in which higher proportion of atretic follicles was registered and a global follicular counting diminuished at the end of the culture, for the A group.

The total population counting of follicles included the degenerate follicles and still the number of follicles observed along the culture decrease for the three culture media showing a described phenomenon and that happens *in vivo* too, the reabsorption by the stromal tissue of the atretic follicles.

The expression of p53 revealed that cells present DNA damage and as cell cycle regulator the overexpression of this protein will induce the arrest of the cell cycle at the G1 phase, inducing the cells arrest development and death.

Our study reveals a variable expression of p53 over the time culture and the different culture media, but the expression of this protein indicates that DNA damages were present. For this marker the G group exhibits the lower proportion of positive cells, in the presence of the higher number of cells and follicles, evidencing that maybe with this medium the cells have less DNA anomalies and consequently lower expression of p53. A positive marking was also seen in oocyte nucleolus, cells of the stroma and cells of the vessels, showing that not only the follicular cells are damaged but also the other type of cells. The obtained results differs of those obtained by Depalo (Depalo et al., 2003), that concludes in her study that the apoptosis process in the earliest stages of follicular development is a cell autonomous physiological event independent of changes in the ovarian medium and not as a consequence of changes or alterations in the cell genome and that occurs first in the GCs and then in the oocyte. However her study was in not cultured fresh ovarian tissue, thus the present results obtained might proved that culture changes cell genome.

The Bcl2 is an anti-apoptotic regulator, providing protection against cell death and have normally an inverse expression of p53 (Depalo et al., 2003). So its absence reveals that those cells have none response capacity to avoid the cell death, sentencing cells to death. This corroborates the finding results of Depalo that demonstrate that Bcl2 was not expressed in the GCs of primordial and primary follicles.

The Ki67 is a nuclear antigen expressed in all phases of the cell cycle except G0, so Ki67 expression reveals cell division activity, so it was expected that during the culture his expression increase, which didn't occur in our study. The expression of Ki67 to the contrary decreased along the time for all culture media. The staining of stroma cells were low, indicating that proliferation of this tissue didn't happen, and that it was probably due to the culture medium, since Fabbri (Fabbri et al., 2003) proved that cryopreservation didn't change capacity of cell proliferation expression with Ki67.

The proportion of p53 cells positively marked was higher than the proportion of Ki67 cells positively marked demonstrating that in our cultures apoptosis was in higher proportion that cell proliferation.

In this present work the results obtained were very different of those obtained for fresh tissue, using similar protocols (Telfer et al., 2008; Hreisson et al., 2002), since both obtained secondary developed follicles after 6 and 7 days of culture respectively. Thus it seems that culture of cryopreserved tissue has different evolution and maybe different needs from those of fresh tissue. On the other hand comparative studies have demonstrated no significant difference in the density and proportion of follicles between fresh and cryopreserved ovarian tissue (Hovatta et al., 1997; Schubert et al., 2005).

The time of culture, the presence or absence of serum and/or a support matrix, effects in ovarian tissue culture have been reported but without consensus of the better system (Hovatta et al., 1997; Hreisson et al., 2002; Sadeu et al., 2008; Telfer et al., 2008). Further studies, specially using cryopreserved ovarian tissue are needed to efficiently the culture and the development of primordial follicles.

CONCLUSION AND PERSPECTIVES

The cortical tissue contains a non renewable quantity of follicles and their oocytes. For women treated for cancer and which fertility is in danger, culturing this tissue under conditions that promote viable mature follicles without risking reintroduction of malignant cells is an very important goal.

At the moment several studies allowed follicular development *in vitro*, at least of a determinant stage of development to the immediately superior (Roy and Tracy, 1993; Abir et al., 1997; Hovatta et al., 1997, 1999; Abir et al., 1999; , Hreinsson et al., 2002; Scott et al, 2004).

The primordial recruitment and his development into the Graafian stage, changes of requirements of the cells, cellular interactions and morphogenesis are major challenges for contemporary cell culture technology.

With this work we show, that after thawing, a decrease of follicular population for the three culture medium, and a higher proportion of degenerating follicles in the Activin A group, in the opposite the GDF-9 group present the lower proportion, confirming the GDF-9 survival role in culture, results corroborate by the lower proportion of GCs positively marked for apoptotic marker p53.

In the opposite we observed that activin A seems to have a negative effect in the earliest stage of follicular development in human.

Further studies for improve the culture medium with the proteins used in the present study and with others as kit Lingand, adapted to the different time of follicular development are pertinent as well as different types of culture.

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APPENDIX

CHEMICALS

Chemical	Supplier	Catalog Nº
Activin A	R&D Systems	338 AC
Ascorbic acid	Sigma	A4403
BSA	Sigma	A9418
DMSO	Sigma	D2650
FCS	EuroBio	CVFSVF040U
GDF 9	CliniSciences	4872
HEPES	EuroBio	CSTHP000P
Insulin	Sigma	I2643
L-15	EuroBio	CM1L15000U
McCoy's 5a	Sigma	M8403
PBS	EuroBio	CS1PBS000U
PSG	Sigma	G6784
Pyruvate sodium	Sigma	P5280
Selenium	Sigma	S5261
Sucrose	Sigma	S7903
Transferrin	Sigma	T8158

The powder chemicals were solubilized with PBS

SOLUTIONS

THAWING SOLUTIONS

	L15	DMSO	SVF	Sucrose
Solution 1	3,9 mL	0,5 mL	0,5 mL	0,1 mL
Solution 2	4,0 mL	0,4 mL	0,5 mL	0,1 mL
Solution 3	4,2 mL	0,2 mL	0,5 mL	0,1 mL
Solution 4	4 mL		1 mL	

DISSECTION MEDIUM

	Initial concentration	Concentration needed	Vol/mL
L-15			910 µL
BSA	40 mg/mL	3 mg/mL	75 µL
Pyruvate sodium	1 M	2mM	2 µL
PSG			10 µL
Ascorbic acid	10 mg/mL	50 µg/mL	5 µL

CULTURE MEDIUM

	Initial concentration	Concentration needed	Vol/ mL
McCoy's			950 µL
BSA	40 mg/mL	0,1%	1 µL
HEPES	1 M	20 mM	20 µL
PSG			15 µL
Insulin *	2 mg/mL	10 ng/mL	1µL
Transferrin *	10 mg/mL	2,5 µg/mL	5 µL
Selenium *	1 mg/mL	4 ng/mL	2 µL
Ascorbic acid	10 mg/mL	50 µg/mL	5 µL
Activin A	10 µg/mL	100 ng/mL	10 µL
GDF 9	100 µg/mL	200 ng/mL	2 µL

*For those reagents dilutions were made due to the lower volume needed

SLIDES READING TABLES

Date mise en culture : Jour de fixation :

Nº DE LA LAME :

HES

ASPECT DU CYTOPLASME	NUCLEOLE	CHROMATINE	Nº CELL. FOLLI.	TYPE DE FOLLICULE

COUPES CYTOPLASMIQUES	COUPES POLAIRES	FOLL. DEGEN.	ETAT DES PAROIS VASCULAIRES	ASPECT DU STROMA

OBSERVATIONS :

Date mise en culture :

Jour de fixation :

Nº DE LA LAME:

P53

TYPE DE FOLLICULE	NOYAU	N° CELL. FOLLI.	
		+	-

	CELLULES /200	
	+	-
STROMA		

	CELLULES /100	
	+	-
VAISSEAUX		

OBSERVATIONS:

Date mise en culture :

Jour de fixation :

Nº DE LA LAME:

Ki 67

TYPE DE FOLLICULE	NOYAU	N° CELL. FOLLI.	
		+	-

	CELLULES /200	
	+	-
STROMA		

	CELLULES /100	
	+	-
VAISSEAUX		

OBSERVATIONS:

Date mise en culture :

Jour de fixation :

Nº DE LA LAME:

Bcl 2

TYPE DE FOLLICULE	NOYAU	N° CELL. FOLLI.	
		+	-

	CELLULES /200	
	+	-
STROMA		

	CELLULES /100	
	+	-
VAISSEAUX		

OBSERVATIONS:

AGREEMENT FOR OVARIAN TISSUE CRYOPRESERVATION

For minors patients

Groupe Hospitalier Pitié-Salpêtrière
UF de Biologie de la Reproduction,
Pr Catherine Poirot
83, bd de l'Hôpital de la Pitié-Salpétrière 75013 Paris. Tél : 01 42 17 75 50

FORMULAIRE DE CONSENTEMENT POUR UNE PATIENTE MINEURE DEVANT BENEFICIER D'UNE CRYOCONSERVATION DE CORTEX OVARIEN

Madame, Monsieur,

La maladie de votre fille nécessite un traitement qui peut perturber le fonctionnement des ovaires et en conséquence, la possibilité d'avoir des enfants. Pour préserver sa fonction ovarienne et sa fertilité, nous vous proposons de prélever et de conserver par congélation du tissu ovarien de votre fille.

Actuellement, plusieurs centaines de femmes et d'enfants ont déjà bénéficié d'une congélation de tissu ovarien. Pour le moment, quelques enfants sont nés après décongélation et greffe de tissu ovarien.

Malgré tout, ces techniques sont encore récentes et nous ne pouvons pas garantir leur efficacité.

Après les différentes consultations médicales, si vous acceptez la cryoconservation du tissu ovarien de votre fille, les étapes spécifiques à cette prise en charge seront les suivantes:

- un prélèvement de sang pour un bilan sérologique (hépatite B, hépatite C, VIH 1 et 2, syphilis) et pour la préparation du milieu de congélation,
- une intervention chirurgicale sous anesthésie générale pour effectuer le prélèvement de tissu ovarien,
- ensuite le tissu ovarien sera préparé, congelé et conservé dans l'azote liquide jusqu'à utilisation,
- au moment de cette cryoconservation, un examen microscopique sur un fragment du tissu permettra d'identifier les différentes cellules présentes.

Nous soussignés,

Madame, ……..……..……..…..née le ……..à……..…..……..

Monsieur, …….…….……..…….né le …….……..à……..

Parents ou tuteur légal de : ……née le :…….à……...
demeurant…….……..……
……...
……
……..

certifions accepter une cryoconservation du tissu ovarien de notre fille conformément à l'article L.2141-11 de la loi n° 2004-800 du 06/08/2004 et l'article R.2142-29, décret n° 2006-1660 du 22/12/2006, avoir été informés et accepter les points suivants :

- Nous avons bien compris que le laboratoire ne peut pas garantir la capacité du tissu ovarien à retrouver ses fonctions,
- La conservation du tissu ovarien de notre fille est renouvelable chaque année sur demande écrite. En conséquence, nous recevrons annuellement un courrier pour exprimer notre souhait de prolonger ou non la conservation, et nous nous engageons à y répondre.
- Nous nous engageons à signaler tout changement d'adresse.

La restitution du tissu ovarien de notre fille ne s'effectuera qu'en sa présence et avec son accord. Le type d'utilisation sera défini après concertation multidisciplinaire entre les différents médecins impliqués dans sa prise en charge (l'oncologue, l'hématologue et les gynécologues obstétriciens, les chirurgiens, les biologistes de la reproduction...).

Dans l'hypothèse où notre fille n'aurait plus besoin de son tissu ovarien ou en cas de décès [(1)] :

[(1)] veuillez cocher la case correspondant à votre choix

□ Nous souhaitons que son tissu ovarien soit détruit

□ Nous autorisons l'utilisation de son tissu ovarien à des fins de recherche

Les informations concernant notre fille seront collectées et feront l'objet d'un traitement informatisé. Ces données demeurent strictement confidentielles et leur consultation ne sera faite que par des personnes astreintes au secret professionnel.

Nous bénéficions, en outre, d'un droit d'accès et de rectification, aux informations la concernant (article 40 de la Loi "Informatique et Liberté"), celui-ci s'exerçant à tout moment, par l'intermédiaire du médecin de notre choix. Nous disposons également d'un droit d'opposition à la transmission des données couvertes par le secret professionnel.

Fait à …….. le……

Le père de la patiente ou le tuteur titulaire de l'autorité parentale

Père, tuteur de l'enfant Mademoiselle…….

Nom……..

Prénom……..

Signature obligatoire* :

La patiente mineure

Nom ……

Prénom……

Signature* :

La mère de la patiente

titulaire de l'autorité parentale

Madame,

Nom…….

Nom……..

Prénom……..

Prénom……..

Signature obligatoire* :

Le médecin biologiste de la reproduction,

Docteur, Professeur

Mademoiselle

Signature obligatoire :

* Signature précédée de la mention *« lu et approuvé »*

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Prénom……..

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La patiente mineure

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Prénom……

Signature* :

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titulaire de l'autorité parentale

Madame,

Nom…….

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Le médecin biologiste de la reproduction,

Docteur, Professeur

Mademoiselle

Signature obligatoire :

* Signature précédée de la mention *« lu et approuvé »*